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Plasma membrane dynamics and tetrameric organisation of ABCG2 transporters in mammalian cells revealed by single particle imaging techniques

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ABSTRACT

ABCG2 is one of three human ATP binding cassette (ABC) transporters involved in the export from cells of a chemically and structurally diverse range of compounds. This multidrug efflux capability, together with a broad tissue distribution in the body, means that ABCG2 exerts a range of effects on normal physiology such as kidney urate transport, as well as contributing towards the pharmacokinetic profiles of many exogenous drugs. The primary sequence of ABCG2 contains only half the number of domains required for a functioning ABC transporter and so it must oligomerise in order to function, yet its oligomeric state in intact cell membranes remains uncharacterized. We have analysed ABCG2 in living cell membranes using a combination of fluorescence correlation spectroscopy, photon counting histogram analysis, and stepwise photobleaching to demonstrate a predominantly tetrameric structure for ABCG2 in the presence or absence of transport substrates. These results provide the essential basis for exploring pharmacological manipulation of oligomeric state as a strategy to modulate ABCG2 activity in future selective therapeutics.

1. Introduction

Eukaryotic multidrug efflux pumps have physiological relevance in metabolite and xenobiotic export, in cancer multidrug efflux and in influencing pharmacokinetics profiles of other pharmaceutical drugs. In humans, at least three members of the ATP binding cassette (ABC[1]) transporter superfamily are capable of multidrug efflux – ABCB1 (P-glycoprotein), ABCC1 (multidrug resistance protein-1) and ABCG2 (breast cancer resistance protein) [1]. The polypeptide chains of ABCB1 and ABCC1 contain the four core domains expected for a functional ABC transporter [2], i.e. two nucleotide-binding domains (NBDs) and two (or three in the case of ABCC1) transmembrane domains (TMDs). ABCG2's primary sequence is different in two significant respects, which make any structural inferences from our knowledge of other ABC transporters difficult [3]. Firstly, the protein has a single NBD and a single TMD in the polypeptide, leading it to be known as a "half-transporter" [4]. Secondly these domains are in the "reverse order" compared to the majority of ABC transporters, with the NBD being N-terminal and the TMD being C-terminal.

Having only half the number of domains required for a functional ABC transporter has led to the assertion that ABCG2 must *at least* dimerise in order to function. Knowledge of its oligomeric state is therefore an essential element in our understanding of ABCG2 function.

To date, however, biochemical and structural analyses of ABCG2 have presented conflicting evidence of its oligomeric organisation. Dimer, tetramer, octamer and dodecamer formation of ABCG2 molecules have all been suggested [5–7], but these studies have often been limited by the need to extract ABCG2 from its membrane environment. Whilst oligomerisation of ABCG2 has been demonstrated in whole cells using FRET (fluorescence resonance energy transfer) or BiFC (bimolecular fluorescence complementation) microscopy [8,9], neither of these techniques are able to distinguish between dimers and higher order oligomerisation. There is, therefore, a pressing need to address ABCG2 stoichiometry in the intact membrane context, not only to improve understanding of the functional basis of this transporter family, but also to open up new pathways to selective inhibitor development, which target ABCG2 protein-protein interactions [10], instead of its less selective drug binding sites [11].

With this ambition in mind, two fluorescence techniques have been increasingly used to determine membrane protein oligomerisation in mammalian cells, with potential to resolve higher order protein complexes. The first, fluorescence correlation spectroscopy (FCS),

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[1] Abbreviations: ABC – ATP binding cassette; AOTF – acousto-optic tunable filters; BiFC – bimolecular fluorescence complementation; EMCCD – electron multiplying charge couple device; FCS – fluorescence correlation spectroscopy; FRAP – fluorescence recovery after photobleaching; FRET – fluorescence resonance energy transfer; GFP – green fluorescent protein; HEK – human embryonic kidney; MX – mitoxantrone; NBD – nucleotide binding domain; PBS – phosphate buffered saline; PCH – photon counting histogram; TIRF – total internal reflection fluorescence; TMD – transmembrane domain.

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records fluorescence fluctuations generated by the diffusion of the fluorescently-tagged protein through a stationary confocal volume, illuminating a focal point of ~0.2 μm^2 on the membrane [12]. Analysis of the fluctuation records with respect to mean amplitude, rather than time, generates a photon counting histogram (PCH) [13], from which the molecular brightness of the fluorescence particles can be derived to indicate oligomerisation [14–16]. In the second, single fluorescent particles are resolved using total internal reflection fluorescence (TIRF) imaging, which provides improved resolution of the lower plasma membrane fluorescent complexes by limiting the depth of excitation field (~100 nm). The amplitudes of these particles are monitored over time in response to high intensity illumination, and the bleaching of individual subunits within the complex is detected as a series of discrete steps. This analysis was recently established as a valuable tool to determine the subunit composition of membrane proteins expressed in *Xenopus laevis* oocytes [17–19] and mammalian cells [20–23].

In the current study, we apply both FCS/PCH and stepwise photobleaching approaches to determine the membrane dynamics and oligomeric state of GFP-tagged ABCG2 expressed in human origin HEK293T cells. These data from independent fluorescence techniques provide novel evidence demonstrating tetrameric organisation of GFP-ABCG2 *in situ* in whole cells.

2. Materials and methods

2.1. Molecular biology and cell culture

An enhanced GFP template containing additional "superfolder" mutations M135T, V163A, S30R, and Y30N [24], and the A206K mutation to prevent fluorescent protein dimerisation [25] was employed. The GFP sequence was inserted in frame at the N-terminus of the ABCG2 cDNA in pcDNA3.1zeo (Invitrogen, Paisley, UK; [8]), using *XhoI* and *XbaI* restriction sites. CD28 and CD86 were amplified from vectors kindly provided by Professor Simon Davis (University of Oxford, UK) with *BamHI*/*XhoI* (CD86) or *EcoRI*/*XhoI* (CD28) flanking restriction sites, and stop codon removal. Both CD28 and CD86 had C-terminal truncations (at residue arginine 185 and arginine 277 respectively) to avoid potential cytoplasmic interactions that could affect the oligomeric behaviour of the controls [26]. Digested PCR products were inserted into pcDNA3.1zeo containing GFP between *XhoI*/*XbaI*, creating CD28-GFP or CD86-GFP fusion protein cDNAs. The tandem GFP-GFP construct contained two repeated GFP cDNAs joined in frame by a two-amino acid linker (Leu-Glu, *XhoI* site). The identities of all constructs were confirmed by DNA sequencing.

HEK293T cell passaging and transfection protocols were as described [8]. Stable cell lines expressing CD28-GFP, CD86-GFP or GFP-ABCG2 were selected using 200 μg/ml zeocin for 10–15 days until healthy colonies were observed, and subsequently maintained at 40 μg/ml zeocin. Mixed populations were also dilution cloned and screened for GFP fluorescence, to allow selection of low expressing cell lines (FCS/PCH and TIRF studies), through intensity comparisons under identical acquisition conditions.

2.2. Mitoxantrone accumulation assay

Stable, mixed population HEK293T cells expressing GFP-ABCG2 were seeded on poly-L-lysine coated 96-well plates (655,090, Greiner Bio-One, Stonehouse, UK). When confluent, cells were incubated with 4 μM mitoxantrone (MX; Sigma-Aldrich, Poole, UK) alone, or in the presence of ABCG2 inhibitor, 1 μM Ko143 [27] Sigma-Aldrich), for 0–60 min. Following fixation with 4% paraformaldehyde in PBS (10 min, room temperature), cell nuclei were counterstained with Hoechst33342 (Invitrogen, 2 μg/ml in PBS). Cell images were acquired (4 sites per well) using an ImageXpress Micro platereader (Molecular Devices, Wokingham, UK), equipped with a 20× Nikon extra-long working distance air objective and standard DAPI (nuclear stain detection), FITC (GFP detection), and Cy5 (MX detection) filter sets as described previously [8]. Average cellular MX fluorescence intensities were calculated by analysing the fluorescence images using the Multi-wavelength Cell Scoring analysis in MetaXpress 5.3 software (Molecular Devices, Wokingham, UK). Data are presented as an average % of MX accumulation (normalised against the highest fluorescence intensities obtained in each experiment) over time in the presence or absence of Ko143.

2.3. Fluorescence correlation spectroscopy (FCS) and photon counting histogram analysis (PCH)

Low expressing stable (CD86-GFP, CD28-GFP, and GFP-ABCG2) or transiently transfected (GFP and tandem GFP-GFP) cells were seeded and grown on poly-L-lysine coated Nunc LabTek 8-well chambered cover glasses. Cells were washed with Hank's balanced salt solution (HBSS) twice and allowed to equilibrate to 22 °C. For FCS measurements acquired on a Confocor 2 (Carl Zeiss GmBH, Jena, Germany), the confocal volume was positioned at the upper plasma membrane of the cells by performing a z-scan at low 488 nm Argon laser power (~0.05 kW/cm^2). FCS measurements used 0.25 kW/cm^2 laser power in a single acquisition divided into 3 × 15 s traces, after a 10 s pre-bleach with 0.60 kW/cm^2. Emitted fluorescence was collected via a 505–550 nm bandpass filter. Cells with <100 kHz average fluorescence intensity were selected to produce autocorrelation decay curves. The autocorrelation function G(τ) was calculated by considering fluorescence intensity fluctuations (δI) from mean intensity <I>, using Eq. (1) – comparing time-points at time t and t + τ for a range of τ values. For cytoplasmic GFP and tandem GFP-GFP, autocorrelation curves were fitted using a one-component 3D model (Eq. (2)). For the membrane proteins ABCG2, CD86 and CD28, autocorrelation curves were fitted with a two-component 2D diffusion models (Eq. (3), with a fast autocorrelation component (τ_{D1} ~ 200–400 μs) assumed to be a product of FP photophysics [28,29]. This allowed derivation of the number (N) and dwell times (τ_D) of the fluorescent particles within the confocal volume. A pre-exponential term (not shown in Eqs. (2) and (3) but described in [28]) was also used to describe other high frequency autocorrelation component (1–10 μs) arising from fluorophore photophysics.

$$G(\tau) = 1 + \frac{<\delta I(t).\delta I(t+\tau)>}{<I>^2} \quad (1)$$

$$G(\tau) = 1 + A\,\frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left(1+\frac{\tau}{S^2\tau_D}\right)^{-0.5} \quad (2)$$

$$G(\tau) = 1 + A\,\frac{1}{N}\left(F_1\left(1+\frac{\tau}{\tau_{D1}}\right)^{-1} + F_2\left(1+\frac{\tau}{\tau_{D2}}\right)^{-1}\right) \quad (3)$$

Autocorrelation curves were only analysed if they reached a clear asymptote as G(τ) approached 1; typically this key criteria in FCS analysis rejects measurements affected by low frequency random movement of plasma membrane, photobleaching during acquisition, or large aggregates of fluorescent particles diffusing through the confocal detection volume. On each experimental day Rhodamine 6G (20 nM) calibration experiments were performed prior to cellular measurements to determine the radius of the confocal volume (r) of the 488 nm laser employed for the excitation of GFP, as described previously [29]. From this assessment of the confocal radius r, diffusion coefficients (D) of the membrane localised fusion proteins were estimated using τ_{D2}, according to $D = r^2/4\tau_{D2}$.

PCH analysis was performed on all the FCS traces accepted for autocorrelation analysis, using single-component (for GFP, tandem GFP-GFP, CD86-GFP) or two-component (for CD28-GFP, GFP-ABCG2) PCH models. These models derived an additional estimate of the number of